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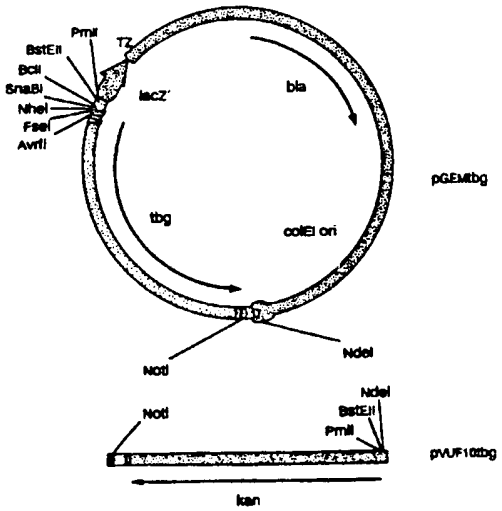

(57) Abstract: The present invention relates to a system for identifying, isolating and utilizing promoter elements useful for expression of nucleotide sequences and the proteins encoded thereby in a thermophile. In one embodiment, a recombinant DNA molecule is provided, and comprises a reporter sequence, a putative thermophile promoter, a selectable marker sequence, and a 3' and a 5' DNA targeting sequence that are together capable of causing integration of at least a portion of said DNA molecule into the genome of a thermophile. Further, within the recombinant DNA, the reporter sequence is under the transcriptional control of a promoter which functions in a thermophile to form a promoter/reporter cassette, the promoter/reporter cassette is flanked by said 3' and said 5' DNA targeting sequences, and the promoter/reporter cassette is positioned in the opposite orientation of the DNA targeting sequences.

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THERMUS PROMOTERS FOR GENE EXPRESSION

FIELD OF THE INVENTION

The present invention relates to the identification and utilization of promoters for expression of nucleic acid sequences in thermophiles.

BACKGROUND OF THE INVENTION

The high-temperature operating conditions of certain industrial processes in the areas of pharmaceutical synthesis, biodegradation of complex agricultural and industrial waste compounds, and food processing dictate the use of thermostable enzymes that can function at high temperatures. A significant advantage thermostable enzymes provide are cost savings resulting from longer storage stability and the higher activity at high temperature.

Thermostable enzymes have traditionally been used for saccharification in food processing and proteolysis in detergent industry (Williams, R. A. D. Biotechnological applications of the genus *Thermus* . Thermophiles: Science and Technology, Reykjavik, Iceland, 1992). Glucosidases are utilized extensively throughout the starch processing industry. Thermostable carbohydrases are directly involved in the manufacture of all starch-derived products. Isomersases are involved in production of high-fructose corn syrup. Two other important industrial carbohydrases are the pectolytic enzymes and lactase (Burgess, K. and M. Shaw. In Industrial enzymology. Ed. by T. Godfrey and Reichelt, J., N.Y. p. p. 260. 1983; Bombouts, F. M. and W. Pilnik. In Microbial enzymes and bioconversions. Ed. by A. H. Rose, N.Y. p. 269. 1980). A recent development in the industrial enzyme area is the use of cellulase for the production of glucose from cellulose (Mandels, M. In Annual reports on fermentation processes. Ed. by G. T. Tsao, N.Y. p. 35. 1982). Proteolytic enzymes, constituting a significant segment of the total industrial enzyme market are utilized in the detergent industry (Godfrey, T. and J. Reichelt. (1983) Industrial enzymology.).

One of the most promising new applications of thermostable enzymes is in the manufacture of specialty chemicals and pharmaceutical intermediates. Enzymes (or biocatalysts) are now being viewed as clearly superior in cases where stereospecific synthetic reactions are involved, such as synthesis of chiral compounds as pharmaceutical intermediates. Enzymes can carry out the reaction more specifically and under conditions which are safer for the environment. Thermostable enzymes have advantages since they are generally more stable in organic solvents, can carry out reactions at high temperatures where substrate and product solubility is higher, and can be recycled and used for longer periods of time because of their inherent stability.

A relatively new application of thermostable enzymes is PCR-based diagnostics. Thermostable polymerases have been extremely useful in the detection and molecular characterization of agents causing cancer, AIDS, and numerous other infectious diseases. Thermostable DNA-polymerase can already compete in market value with enzymes having

traditional applications. Thermostable DNA replication proteins have important applications in molecular biology research.

A significant limitation to the widespread use of thermostable enzymes in such applications is the difficulty in expressing the proteins in a host bacteria. Thermophilic bacteria belong to a wide range of very different taxonomic groups (Kristjansson, J. K. and K. O. Stetter. Thermophilic bacteria. In Thermophilic bacteria. Ed. by J. K. Kristiansson, CRC Press, Inc., Boca Raton. p. 1-18. 1992). One of the best studied is the gram negative genus *Thermus*. Species belonging to this genus are easy to handle, growing aerobically in a broad temperature range of 45 to 85 °C and not requiring pressurized incubation devices (Williams, R. A. D. Biotechnological applications of the genus *Thermus*. Thermophiles: Science and Technology, Reykjavik, Iceland, 1992). These strains grow to high densities in simple and inexpensive liquid media and form colonies on solid agar. With a doubling time less than 2 hours, the microorganisms of the genus *Thermus* are suitable organisms for various industrial applications.

Despite the widespread interest in *Thermus* cultures for a variety of applications, there is a need for systems to control gene expression in *Thermus*. Currently the expression of heterologous genes in thermophilic hosts is difficult and inconvenient. Expression vectors for thermophiles do not provide a choice of promoters or ribosome binding sites, nor do they provide a convenient ways to regulate expression. The reagents and methodologies provided herein allow for the production of commercially important enzymes including those from hyper- and extreme-thermophiles that can be difficult to produce using other systems. Also provided are reagents and methodologies for the construction of high-temperature fermentation strains which have been metabolically engineered with exogenous DNA for use in bioprocess applications such as production of complex molecules and pharmaceutical intermediates. Additionally, the systems provided herein are useful for thermostabilization of mesophilic proteins by genetic selection in a thermophile, which can also lead to altered enzymatic activity and a better understanding of the biochemical determinants for thermostability.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. Construction of an integrative promoter probe vector to look for Thermus promoters in *E. coli*.

FIGURE 2. Homology comparison of VV57 with the *T. thermophilus* chaperonin-10 and chaperonin-60 promoter region. The sequence derived from the VV57 promoter clone insert was subjected to BLASTN analysis and the resulting homology comparisons were analyzed. The VV57 sequence matched the promoter region containing the -35, -10 promoter signal sequences as well as the ribosomal binding site.

FIGURE 3. Construction of pTG200 and development of promoter test vectors. A) Comparison of terminator sequences from *Thermus*. The his terminator was used in the

construction of pTG200. B) pTG200 consists of an *E. coli* shuttle vector with the *Thermus leuB* gene disrupted by the promoterless kantr2 gene in the opposite direction. A strong *Thermus* transcription terminator is placed downstream of the kantr2 gene to prevent transcription through the gene in the opposite direction. Promoter-test vectors were constructed by using primers to the two ends of the kan gene with an extended 50-60 bp promoter attached at the 5'end.

SUMMARY OF THE INVENTION

The present invention relates to a system for identifying, isolating and utilizing promoter elements useful for expression of nucleotide sequences and the proteins encoded thereby in a thermophile. In one embodiment, a recombinant DNA molecule is provided, and comprises a reporter sequence, a putative thermophile promoter, a selectable marker sequence, and a 3' and a 5' DNA targeting sequence that are together capable of causing integration of at least a portion of said DNA molecule into the genome of a thermophile. Further, within the recombinant DNA, the reporter sequence is under the transcriptional control of a promoter which functions in a thermophile to form a promoter/reporter cassette, the promoter/reporter cassette is flanked by said 3' and said 5' DNA targeting sequences, and the promoter/reporter cassette is positioned in the opposite orientation of the DNA targeting sequences. In another embodiment, a method of identifying a thermophile promoter comprising transforming a thermophile with the above-described recombinant DNA molecule detecting expression of the reporter sequence is provided. The present invention also relates to promoters which have been identified by the above method.

DETAILED DESCRIPTION

Within this application, unless otherwise stated, definitions of the terms and illustration of the techniques of this application may be found in any of several well-known references such as: Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989); Goeddel, D., *ed.*, *Gene Expression Technology*, *Methods in Enzymology*, **185**, Academic Press, San Diego, CA (1991); "Guide to Protein Purification" in Deutshcer, M.P., ed., *Methods in Enzymology*, Academic Press, San Diego, CA (1989); and, Innis, et al., *PCR Protocols: A Guide to Methods and Applications*, Academic Press, San Diego, CA (1990). The references, issued patents and pending patent applications cited herein are incorporated by reference into this application.

For the purposes of this application, a *promoter* is a regulatory sequence of DNA that is involved in the binding of RNA polymerase to initiate transcription of a gene. A "thermophile promoter" is a promoter that functions in a thermophilic organism such as *Thermus*. A *gene* is a segment of DNA involved in producing a peptide, polypeptide or protein, including the coding region, non-coding regions preceding ("leader") and following ("trailer") the coding region, as well as intervening non-coding sequences ("introns") between

individual coding segments ("exons"). Coding refers to the representation of amino acids, start and stop signals in a three base "triplet" code. Promoters are often upstream (" '5 to") the transcription initiation site of the corresponding gene. Other regulatory sequences of DNA in addition to promoters are known, including sequences involved with the binding of transcription factors, including response elements that are the DNA sequences bound by inducible factors. Enhancers comprise yet another group of regulatory sequences of DNA that can increase the utilization of promoters, and can function in either orientation (5'-3' or 3'-5') and in any location (upstream or downstream) relative to the promoter. Preferably, the regulatory sequence has a positive activity, i.e., binding of an endogeneous ligand (e.g. a transcription factor) to the regulatory sequence increases transcription, thereby resulting in increased expression of the corresponding target gene. A promoter may also include or be adjacent to a regulatory sequence known in the art as a *silencer*. A silencer sequence generally has a negative regulatory effect on expression of the gene.

Provided herein are methodologies and reagents for obtaining and utilizing expression systems for efficient expression of nucleotide sequences in a host organism. Preferably, the host cell is a member of the kingdom Bacteria, Archea or Eukarya. In one embodiment, and for the purpose of testing the constructs provided herein, it is preferred that the host cell is *E. coli*. More preferably, it is preferred that the expression systems comprise promoter elements capable of regulating gene expression in a thermophile. Even more preferably, the host cell is a member of the genus *Thermus*, and most preferably the host cell is of the species *Thermus flavus*, *Thermus thermophilus*, *Thermus aquaticus* or other related species. The instant invention provides reagents and methodologies useful for identifying promoters having activity in a thermophile, preferably of the genus *Thermus*.

Using the reagents and techniques described in this application, inducible and constitutive promoters, integrative and plasmid-based vectors, and nucleic acids containing secretion signals may be isolated. The vectors utilized may be any vector suitable to isolation and characterization of a promoter. For instance, the vectors utilized may be plasmid, bacteriophage, virus, phagemid, cointegrate of one or more species, etc. Preferably, the vector is amenable to expression of a nucleotide sequence in a prokaryotic cell such as *Thermus* or *E. coli*. It is further preferable that the vectors be capable of functioning in different types of cells (ie, shuttle), such as *Thermus* or *E. coli*.

It is also possible to use the present invention to accomplish model pathway engineering and/or thermostabilization using tandem expression systems. The present invention provides promoter sequences and expression vectors that allow for various levels of expression in a thermophilic host cell. The levels of expression may be controlled by the inherent properties of the promoter itself or with the assistance of an additional regulatory sequence. The availability of such promoter sequences will provide a crucial tool for use in driving expression of thermostable enzymes for use in multiple applications.

As would be understood by the skilled artisan, the development of efficient thermophile expression systems is important for several applications including, for example, the isolation and use of nucleic acid sequences from extreme- or hyper-thermophiles that may not be efficiently expressed in mesophilic systems. Thousands of genes and uncharacterized ORFs have been discovered from these industrially important organisms through genome sequencing projects. High-temperature fermentation strains with altered metabolism may be useful in bioprocess applications for the production of pharmaceutical intermediates. The present invention provides tools for thermostabilizing mesophilic proteins by selection in *Thermus* and allows for the identification of the sequence determinants involved in thermostabilization.

In addition, the reagents and methodologies provided herein may be utilized for the following non-limiting exemplary purposes:

1. *Proper expression of extreme and hyper-thermophilic genes.* The discovery of novel genes from genome sequencing projects has significantly expanded the sources of thermostable enzymes. Total genome sequencing projects of extreme and hyper-thermophiles such as *Sulfolobus sulfactericus, Pyrococcus furiosus, Methanobacterium thermoautotrophicum, Thermus flavus* and others are important for the development of new industrial processes where thermostable enzymes may be utilized.

In order to be a commercially viable enzyme, the enzyme must be capable of being produced and recovered in large quantities in an organism with low cultivation cost. A heterologous host is useful because of the difficulties in growing hyperthermophiles and the lack of systems for cloning and gene analysis for such strains. In one embodiment of the present invention, the host cell is *E. coli.* However, in certain situations, *E. coli* is a sub-optimal host for hyperthermophile gene expression. In several studies, it was shown that certain hyperthermophilic proteins could not assemble properly in *E. coli* (Robb, et al. Gene Discovery and Production of Recombinant Gene Expression in the Marine Hyperthermophile *Pyrococcus furiosus.* The 7th International Symposium on the Genetics of Industrial Microorganisms., Montreal, 1994; Laderman, et al. (1993) a-Amylase from the Hyperthermophilic Archaebacterium *Pyrococcus furiosus. J. Biol. Chem.* 268:24402-24407.). In addition, the temperature optima of the proteins being studied are typically too high to permit an analysis of their function *in vivo* in the mesophilic organism. Thus, in a preferred embodiment, the host cell is from the genus *Thermus.* Temperature-dependent folding and activity of proteins from hyperthermophiles make species of the genus *Thermus* a more preferred host for expression of such proteins.

2. *Engineered fermentation strains for high-temperature bioprocesses.* Many industrial bioprocesses utilize whole-cell fermentation techniques. In many instances, the use of an isolated enzyme system is too expensive or impractical. Many enzymes, such as dehydrogenases that may be utilized to carry out chiral synthesis of pharmaceutical intermediates, require co-factors such as NAD(P) to for their reactions. Cofactors are utilized

stoichiometrically during the reaction and must be repeatedly added to the reaction mixture or the reaction must regenerate the cofactor. A whole-cell system provides a alternative for many of these enzymes. Other enzymes may be membrane-bound or require complex subunit or multi-enzyme complexes (such as cytochrome P-450s), allowing for simpler implementation using a whole-cell system. Finally, the synthesis of complex molecules such as steroids, antibiotics, and other pharmaceuticals may require complicated and multiple catalytic pathways. In an isolated system, each step would need to be engineered. In contrast, the organism utilized in a whole cell system provides each of the required pathways. The tools provided herein may be utilized to engineer *Thermus* with multiple genes, thus providing the organism with the necessary pathways for carrying out such bioprocesses.

In addition, it is often desired to carry out synthetic reactions at high temperatures, because either the reaction is exothermic (therefore cooling is not needed), the substrate or product solubility is greater at higher temperature (which can drastically increase throughput), the viscosity of the reaction is improved (as in the case in many food applications such as the processing of cheese whey), or the reaction proceeds significantly faster at the higher temperature.

3. *Genetic thermostabilization of mesophilic genes.* The thermolability of most mesophilic proteins can limit their industrial use. It was proposed in the early 1980s that thermostabilization of mesophilic proteins could be accomplished by carrying out activity selections in organisms which grow at high temperatures (Matsumura, et al. (1984) Enzymatic and Nucleotide Sequence Studies of a Kanamycin-Inactivating Enzyme Encoded by a Plasmid from Thermophilic Bacilli in Comparison with That Encoded by Plasmid pUB110. *J. Bacteriol.* **160**:413-420; Liao, et al. (1986) Isolation of a thermostable enzyme variant by cloning and selection in a thermophile. *Proc. Natl. Acad. Sci. USA.* **83**:576-580). Applicants have developed several directed evolution methods to accelerate the evolution of protein properties such as thermostability through the use of both *in vivo* and *in vitro* techniques. Directed evolution relies on a random, but targeted, approach to generating mutations of interest. By carrying out sequential generations of random mutagenesis on a gene of coupled with selection or screening for the resulting proteins, numerous proteins with improved properties have been developed. In each generation, a single variant is generally chosen as the parent for the next generation, and sequential cycles allow the evolution of the desired features. Alternatively, effective mutations identified during one or more generations can be recombined using methods such as 'DNA shuffling' (represented by, for example, sexual PCR). Traits which have been enhanced may include but are not limited to improved substrate specificity, catalytic activity, activity in the presence of organic solvents, expression level and stability.

Liao, et al. ((1986) Isolation of a thermostable enzyme variant by cloning and selection in a thermophile. *Proc. Natl. Acad. Sci. USA.* **83**:576-580) first demonstrated *in vivo* thermostabilization of a gene by using kanamycin nucleotidyl transferase in *Bacillus*

stearothermophilus where resistance to 63°C was shown. To improve the genetic thermostabilization approach, a gene transfer system for *Thermus* was developed where the upper growth limit was above 80°C instead of 65°C as in *Bacillus* (described in, for example, US 5,786,174 which is hereby incorporated by reference). These experiments were initially conducted using the thermostabilized *kan* gene, in which the initial Km^r supported growth only to 55°C in *Thermus* and not to 63°C as reported by Liao, et. al. ((1986) Isolation of a thermostable enzyme variant by cloning and selection in a thermophile. *Proc. Natl. Acad. Sci. USA*. **83**:576-580). The regulated expression system provided herein allows for fine-tuning of thermostabilization selection experiments so that the temperature range can be regulated and controlled and cutoff temperatures for selection adjusted in subsequent rounds of mutagenesis.

Some important elements of *Thermus*' genetic background have been previously described. The generation of mutations (Koyama, et al. (1990) Cloning and sequence analysis of tryptophan synthetase genes of an extreme thermophile, *Thermus thermophilus* HB27: Plasmid transfer from replica-plated *Escherichia coli* recombinant colonies to competent *T. thermophilus* cells. *J. Bacteriol.* **172**:3490-3495; Koyama, et al. (1990) A plasmid vector for an extreme thermophile, *Thermus thermophilus*. *FEMS Microbiol. Lett.* **72**:97-102; Lasa, et al. (1992) Insertional mutagenesis in the extreme thermophilic eubacteria *Thermus thermophilus* HB8. *Molec. Microbiol.* **6**:1555-1564), chromosomal integration (Koyama, et al. (1990) Cloning and sequence analysis of tryptophan synthetase genes of an extreme thermophile, *Thermus thermophilus* HB27: Plasmid transfer from replica-plated *Escherichia coli* recombinant colonies to competent *T. thermophilus* cells. *J. Bacteriol.* **172**:3490-3495; Koyama, et al. (1990) A plasmid vector for an extreme thermophile, *Thermus thermophilus*. *FEMS Microbiol. Lett.* **72**:97-102; Lasa, et al. (1992) Insertional mutagenesis in the extreme thermophilic eubacteria *Thermus thermophilus* HB8. *Molec. Microbiol.* **6**:1555-1564), plasmids (Mather, et al. (1990) Plasmid-associated aggregation in *Thermus thermophilus* HB8. *Plasmid*. **24**:45-56; Hishinuma, et al. (1978) Isolation of extrachromosomal deoxyribonucleic Acids from extremely thermophilic bacteria. *Jour. of General Microbiology*. **104**:193-199.), and phages (Sakaki, et al. (1975) Isolation and Characterization of a Bacteriophage Infectious to an Extreme Thermophile, *Thermus thermophilus* HB8. *J.Virol.* **15**:1449-1453) have also been studied. Several successful attempts to develop cloning systems using plasmids and chromosomal integration systems were demonstrated (Koyama, et al. (1986) Genetic transformation of the extreme thermophile *thermus thermophilus* and of other *thermus* spp. *J. Bacteriol.* **166**:338-340; Lasa, et al. (1992) Development of *Thermus-Escherichia* Shuttle Vectors and Their Use for Expression of the *Clostridium thermocellum celA* Gene in *Thermus thermophilus*. *J. Bacteriol.* **174**:6424-6431; Mather, et al. (1992) Development of Plasmid Cloning Vectors for *Thermus thermophilus* HB8: Expression of a Heterologous, Plasmid-Borne Kanamycin

Nucleotidyltransferase Gene. *Appl. Environ. Microbiol.* **58**:421-425.). However, none of these provide the versatility as those provided herein.

4. *Thermus expression signals*. More than twenty genes from *Thermus* species have been cloned and sequenced (**Table 1**). However, none of these sequences illustrate the optimal regulatory elements needed to develop a useful system for expression in a thermophile such as *Thermus*. Applicants have previously documented the nucleotide sequences encoding phosphatases, glycolytic enzymes (ß-glucosidases and ß-galactosidases), and biosynthetic genes (*pyrE, hisB, leuB*) from *Thermus* by complementation of their functions or by testing the specific activities (Weber, et al. (1995) A chomosome integration system for stable gene transfer into *Thermus flavus*. *Bio/Technology*, Vol. 13(3): 271-275). Expression of these sequences in *E. coli* demonstrated that most *Thermus* genes are capable of being transcribed in *E. coli*. The comparison of nucleotide sequences from some *Thermus* genes and operons reveals putative translation initiation signals that are similar to known *E. coli* motifs (**Table 1** shows exemplary sequences).

Table 1

***Putative translation initiation signals of known genes of the genus* Thermus**

Organism	Gene	Start codon	RbS	-10 region	-35 region	Ref.
T.aquaticus YT1	L-lactate DH	GTG	n.f.	n.f.	n.f.	(20)
	aqual	ATG	AGGAG	TAGCTT	TTGACA	(21) (22)
T.aquaticus B	*sucD*	GTG	GGAGGTG	n.f.	n.f.	(23)
	mdh	GTG	AAGGAG	n.f.	n.f.	(23)
T.thermophilus HB8	*tufB*	ATG	AGGAGGA	n.f.	n.f.	(24)
	tufA	ATG	AGGAGGA	n.f.	n.f.	(25)
	icdh	ATG	GGAGG	TGTAGT	TTACAA	(26)
	slpA	ATG	AAGGAGGTG	TACGAT	TTGACA	(27)
	Xylose isomerase	GTG	AGGAGG	n.f.	n.f.	(28)
	gltX	ATG	GAGG	ATAAT	n.f.	(29)
	nox	ATG	n.f.	n.f.	n.f.	(30)
	16S RNA			TAGCAT	TTGACA	(31)
	23S/5S RNA			TATCTT	TTGACA	(31)
	NADH dh		AAGGAGGGG	TAAGAT	TTGCGC	
	4.5S RNA			TATACT	TAGCCT	(31)
T.thermophilus HB27	*trpB*	ATG	AGGGAG	TAGGAT	TTTACC	(11)
	trpA	GTG	GGGAGG	n.f.	n.f.	(11)
T.flavus AT62	*sucB*	TTG	AAGGGAGG	TATAAT	n.f.	(32)
	sucA	GTG	GGAGG	n.f.	n.f.	(32)
	mdh	GTG	AAAGGAGG	n.f.	n.f.	(32)
E. coli	*ntsA/intB*	ATG	AAGGAGGTG AAGGG	TATAAT TAGACT	TTGACA TTGTAG	

n.f. - no homology to *E. coli* found; RbS = ribosome binding site; "-10" and "-35" regions indicate initiator regions.

The expression system provided herein comprises certain genetic elements whose configuration has been manipulated to ensure high levels of protein synthesis in a thermophilic host such as *Thermus*. In one embodiment, the central element of the expression system is a promoter positioned upstream of a ribosome-binding site, RBS, which is further under the control of a regulatory gene. Provided herein are several exemplary *T. thermophilus* promoters having activity in *T. flavus*. In another embodiment, novel promoter regions from bacteria belonging to genus *Thermus* are provided. In yet another embodiment, a promoter probe vector for *Thermus* is provided and is useful for evaluating promoter strength.

The isolation of a promoter that functions in a thermophile such as *Thermus* promoters may be accomplished by generating a library of genomic fragments from the

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organism by limited restriction digest or other method for generating DNA fragments and inserting the fragments into a reporter vector. As a source for the genomic DNA, one could use any suitable bacterial strain. Preferably, the strain is of *Thermus*. As an example, in constructing a *Thermus* promoter library, *Thermus* chromosomal DNA may be partially digested with a frequent-cutting endonuclease such as *Sau*3AI and a fraction of the digested fragments having a desired size (for example, approximately 1kb) purified by any suitable method, such as elution from an agarose gel. These fragments are then ligated with DNA from a reporter vector that has been digested with an appropriate enzyme for ligation to the genomic fragment.

To function properly, the reporter or promoter-probe vector requires the following elements: 1) an *E. coli* origin of replication (ColEI was used in pGEMtbg); 2) a marker gene, which functions in *E. coli* (such as the selective drug-resistance marker *bla* that confers ampicillin resistance); 3) a promoterless reporter sequence; and, 4) a transcriptional terminator (TT) upstream of the reporter gene. Other origins of replication or vector systems, marker genes, reporter genes, and terminators can be used as well to construct similar promoter-probe vectors.

The reporter vector may comprise a plasmid backbone derived from a commonly available vector plasmids including but not limited to pBR322, pBR325, pBR327, pUC 8, pUC 9, pUC 41C, pUC18, pUC19, piz 18, and piz 19. The reporter vector is arranged such that a suitable reporter sequence such as *lacZ*, tbg, or a drug resistance gene is positioned downstream (or 3') from a polylinker site containing serveral and/or unique restriction enzyme sites. A suitable reporter sequence encodes a gene product detectable via a colorimetric, chromogenic, fluorometric, enzymatic activity or other assay.

A number of genes can be used as either markers to detect insertion of the gene in *Thermus* or as reporters which can be used to analyze expression in Thermus. **Table 2** contains a few examples of such genes, but it should be understood by the skilled artisan that others may also be suitable. Some of these genes confer selectable phenotypes. In this case, media conditions can be established so that only colonies which have expression of these genes activated will grow. Other genes confer phenotypes which can be screened. A screenable phenotype can often yield information about levels of gene expression.

Table 2

Marker/reporter genes which can be used in Thermus.

Marker	Description	Type	Integrative		
			FN	Comments	Ref
kantr2	Thermostable kanamycin resistance gene	SEL	+	Works with a variety of promoters and successfully used in promoter-test experiments Expression level effects resistance level in host.	A
HLADH	Horse Liver Alcohol Dehydrogenase.	SCR	+	Expression confirmed under control of Leucine promoter in Thermus. Although not from a thermophile, the gene is stable up to about 70°C. Can be quantitated.	B
pyrE	Orotodine-5'-phospho-ribosyl—transferase	SEL	+	Selectable marker which can be used as a site of integration in *Thermus*.	C
leuB	Isopropyl malate dehydrogenase	SEL	+	Selectable marker used as a site of integration in *Thermus*.	D

Abbreviations: SEL: selectable marker; SCR: screenable marker; ND not determined; FN: Functional.
References: A. US 5,786,174; (Weber, et al. (1995) A chomosome integration system for stable gene tranfer into *Thermus flavus*. *Bio/Technology*, Vol. 13(3), pp. 271-275). B. U.S. Prov. App. No. 60/046,182 filed May 12, 1997. C. US 5,786,174. D. US 5,786,174.

The vector may contain more than one reporter sequence located 5' and 3' of the polylinker region. In this manner, the orientation of a promoter region at the polylinker site will not affect expression of the reporter sequence. The genomic fragments are ligated into the polylinker region using standard techniques. In one embodiment, the promoter sequences are amplified by PCR using primers containing, for example, an EcoRI site, -35, -10 and several downstream residues (to include the +1 transcription site). The amplified sequences are then cloned into a TG200 reporter sequence by digesting the PCR fragment with EcoRI and HindIII, followed by subcloning into the pTGeporter vector by digesting the PCR fragment with EcoRI and HindIII.

In a preferred embodiment, a library of *T. thermophilus* chromosomal fragments is generated by restriction enzyme digestion and cloned into a reporter vector. The reporter sequence is positioned downstream (or 3') of the polylinker region such that insertion of a promoter sequence into the polylinker region will result in expression of the reporter sequence.

Following construction of the reporter vector, the vector may then be transformed into a host cell such as *E. coli* and screened for promoter activity. Those fragments showing promoter activity in *E. coli* are then transformed into a thermophile to detect promoter activity in the thermophile. For sequences with promoter activity in *E. coli* identified from a *T. thermophilus* chromosomal fragment library, the reporter vector is preferably subsequently transformed into *T. thermophilus*, and expression of various markers and model enzymes assayed.

In another embodiment, a reporter vector is utilized which has an integrative element capable of driving integration of the reporter sequence into the genome of the host organism. Preferably, the reporter sequence is positioned in the opposite orientation of the integrative sequences such that occlusion transcription does not occur. As a further safeguard, it is preferable that a transcriptional termination (TT) sequence consisting of a inverted repeat with an A/T region comprising a repeat region for pausing an RNA polymerase where AT serves to separate it from the DNA template, be included in the integration sequence. Similar to the reporter vector described above, the reporter sequence is preferably positioned downstream of a polylinker site into which a putative promoter sequence may be inserted. For example, an integrative vector may comprise portions of the *Thermus* leuB gene both 5' and 3' of a cassette containing a drug resistance gene adjacent to a putative promoter sequence. If the putative promoter sequence is capable of driving gene expression in *Thermus*, the host cell will gain resistance to a compound by virtue of expression of the reporter sequence (ie, drug resistance gene) controlled by the putative promoter sequence. The absence of drug resistance indicates that the promoter is not active in *Thermus*.

In another embodiment, sequencing of the *Thermus* genome may be performed and putative promoter sequences identified using computerized searching algorithms. For example, a region of a *Thermus* genome may be sequenced and analyzed for the presence of putative promoters using Neural Network for Promoter Prediction software, NNPP. NNPP is a time-delay neural network consisting mostly of two feature layers, one for recognizing TATA-boxes and one for recognizing so called "initiators", which are regions spanning the transcription start site. Both feature layers are combined into one output unit. These putative sequences may then be cloned into a reporter vector suitable for preliminary characterization in *E. coli* and/or direct characterization in *Thermus*.

To optimize the promoter sequence, the length of the promoter sequence can be optimized by performing deletion analysis, such as by using an endonuclease (such as ExoI or Bal31) to create sequential deletions in the promoter sequence or by generating a series of oligonucleotides with shortened sequences from each side of the isolated promoter sequence. The individual deletions can then be tested for activity and expression from each of the promoter regions can be quantitated to determine the minimal sequence needed to confer expression. This minimal promoter region can then be used to express genes of interest in *Thermus*.

The reagents and methodologies provided herein also provide for the identification of regulated promoters and regulatory elements. By exchanging certain promoter elements from one reporter vector to another using standard molecular biology techniques, specific sequences having certain regulatory effects (ie, increase or decrease expression) on expression of a sequence may be identified. For instance, following identification of a promoter region within a DNA fragment using the techniques described above, certain portions of the promoter may be deleted or excised from the DNA fragment, and the modified

promoter re-tested. In the event that expression is observed after this modification, and determination of whether expression has increased or decreased following modification, a positive or negative regulatory element of the promoter may be identified. In addition, specific regions of the putative promoter element may be isolated and tested in isolation. In this manner, specific elements may be identified that regulate gene expression in the host cell. In addition, various regulatory elements identified as described above may be combined into novel promoter sequences. It is also possible to use the techniques described herein to construct hybrid regulated promoters and vectors for regulated expression by combining one or more regulatory elements with a promoter sequences not typically associated with that regulatory elements. The hybrid promoters can then be tested for activity and expression from each of the promoter regions can be quantitated to determine the minimal sequence needed to confer expression. The hybrid promoter region may then be used to express genes of interest in *Thermus*. Thus, the development of efficient regulated promoters for expression of nucleotide sequence in a thermophile is provided by the instant invention.

Trans-acting regulatory elements may also be identified by screening the libraries in *E.coli*. As will be understood by the skilled artisan, these elements can be placed on different plasmids and both will remain functional.

The constructs described herein may also be utilized to construct optimal expression systems for the production of industrially important thermophilic model proteins including but not limited to lipases, esterases, hydrogenases, and proteases. In addition, the constructs can be utilized to generate bacterial strains with multiple chromosomal insertions and characterize such strains for use in fermentations.

The following Examples are for illustrative purposes only and are not intended, nor should they be construed as limiting the invention in any manner. Those skilled in the art will appreciate that variations and modifications can be made without violating the spirit or scope of the invention.

EXAMPLES

Example 1

Screening a library of *T. thermophilus* chromosomal fragments for sequences with promoter activity in *E. coli*

A. Assembly of a promoter probe vector for selection of *Thermus* promoters in *E. coli.*

One strategy for discovery of *Thermus* promoters is to first identify a promoter from *Thermus* which functions in an intermediate strain, such as *E. coli*, and then test the promoters which have been identified in *Thermus*. Performing this two-step process can potentially dissociate the promoter from a regulatory element and help identify *Thermus* promoters that may be tightly controlled.

For primary selection of *Thermus* chromosomal fragments exhibiting promoter activity in *E. coli*, the promoter probe vectors pGEMtbg and pVUF10tbg (**Fig. 1**) were constructed. The promoter-probe vectors utilized herein include: 1) an *E. coli* origin of replication (ColEI was used in pGEMtbg); 2) a marker gene which functions in *E. coli* (the selective drug-resistance marker *bla* conferring ampicillin resistance was used in pGEMtbg); 3) a promoterless reporter gene (*tbg* was used in pGEMtbg); and, 4) a transcriptional terminator (TT) upstream of the reporter gene.

In the construction of pGEMtbg, the *tbg* gene of *T. aquaticus* encoding Thermo-ß-galactosidase (Tbg) was used as a reporter sequence. Tbg expression can be detected using several possible chromogenic substrates such as 5-Bromo-4-Chloro-3-indolyl-ß-D-galactopyranoside (X-Gal) and 5-Bromo-4-Chloro-3-indolyl-ß-D-glucopyranoside (X-Glc) to identify clones exhibiting ß-glucosidase (or ß-galactosidase) activity. Expression of *E. coli* ß-glucosidase and ß-galactosidase activities are tightly controlled under uninduced conditions. In addition, the background activity of the endogenous enzyme is insufficient to turn colonies blue and *E. coli lacZ* ß-galactosidase mutants are common. Tbg also demonstrates thermostability, which facilitates assay of the enzyme's activity in crude cell lysates. Heating of lysates for 15 minutes at 65°C totally inactivates endogeneous activity, making the detection of low activities of Tbg possible. To incorporate the *tbg* gene into pGEM, it was amplified by PCR.

The *tbg* gene was isolated from a preparation of *Thermus aquaticus* genomic DNA. Primer sequences used for the PCR amplification of the *tbg* gene to construct pGEMtbg included primer 187 which contained a PmlI, BstEII restriction sites followed by the *trp* transcriptional terminator (underlined) followed by BclI, SnaBI, NheI, FscI, AvrII restriction sites, followed by a sequence homologous to the 5' end of the tbg gene (bold) started with a putative ATG site as depicted below. Primer 227 contained sequence homologous to the 3' end of the tbg gene.

187 5'-CACGTGGTTA CCCGCCTAAT GAGCGGGCTT TTTTTTGATC ATACGTAGCT AGCCCCGGCC GGCCTAGGAT **GGCAATTATT CAATTTC**-3' (SEQ ID NO: 1)

227 5'-TTAATATTCA AACCATTTAT TTTCTAT (SEQ ID NO: 2)

The 5' end primer was designed so that *tbg*, upstream of the Shine-Delgarno (SD) site, had unique *Sna*BI and *Bcl*I sites for cloning blunt-ended DNA fragments and fragments obtained as a result of partial digestion with *Sau*3AI. The strong transcription terminator of *E. coli trp* operon was included upstream of the cloning sites. The PCR fragment was subcloned into the pGEM-T vector (obtained from Promega) to generate pGEMtbg using standard techniques. pGEM-T allows direct cloning of PCR products without the need for restriction digestion. Clones with the proper orientation of the gene were determined by restriction analysis.

Plasmid pVUF10tbg (**Fig. 1**) was constructed by inserting the kanamycin drug resistance marker kantr2 (Weber, et al. (1995) A chomosome integration system for stable gene tranfer into *Thermus flavus*. *Bio/Technology*, Vol. 13(3): 271-275) into the Nde I to Not I site of pGEMtbg. The fragment containing the kanamycin gene was prepared by amplification with primers 388 and 442 listed below. The fragment was digested with NotI and NdeI and cloned into pGEM-tbg which had also been digested with NdeI and NotI.

442 Containing the Nde I (bold), NstEII, and PmlI site followed by homology to the kanamycin gene:

5'TGGTTA**CCAT ATG**GTAACCA CGTGAATGGA CCAATAATAATG (SEQ ID NO: 3)

388 Containing the NotI site (bold) followed by the rrnC transcriptional terminator (underlined) and homology to the kanamycin gene:

5'GTTATCTGAAA**GCGGCCGC**TTTCAGATAAAAAAAATCCTTAGCT TTCGCTAAGGATGGATTTCTGGCTCAAAATGGTATGGTTTTGAC-3' (SEQ ID NO: 4)

The resultant PCR fragment was then isolated and inserted into pGEMtbg plasmid using standard molecular biology techniques.

B. Construction and screening of a *T. thermophilus* genomic library in *E. coli*

To construct a *Thermus* promoter library, *T. thermophilus* chromosomal DNA was partially digested with *Sau*3AI and a fraction of the digested fragments of 1 kb size were purified by elution from an agarose gel. These fragments were then ligated with DNA from the pVUF10 cloning vector which has been digested with *Bcl*I for cloning. *T. thermophilus* was used as the source of genomic DNA since the *T. thermophilus* and *T. flavus* strains utilized are highly related, but not identical. The ligated DNA was used to transform *E. coli*. Transformed cells were cultured on LB agar containing X-glc at 50 μg/ml, and cultured for

several days at 37°C. Recombinant clones exhibiting promoter activity (approximately one percent of all recombinant clones) developed color of different shades of blue due to tbg. Out of several hundred of blue colonies, 24 were randomly selected for further analysis.

The nucleotide sequences of each insert was determined. The entire insert was sequenced for clones VV12, VV18, VV51 and VV57 and about 50% of the sequence was determined for the remaining clones. The sequences of clones 1 and 2 overlapped and were combined resulting in the sequence designated VV1-2. Computer analysis of these sequences using BLASTN search algorithm revealed putative core promoter regions showing similarity to the consensus promoter sequence of *E. coli* (**Table 3**). Sequence analysis of promoter VV1-2 revealed two inverted repeats, one of which was at the transcription-start site. These sequences were AT-rich (below 40% GC) compared with the GC-rich content of random *Thermus* DNA which is about 72% GC. This preliminary search was performed using Sequencher DNA assembler (commercially available). One of these potential promoters matched the known promoter for *T. thermophilus* chaperonin (**Figure 3**).

Table 3

Promoters identified from homology search

Sequence	clone
TTGACATTCCCCCCGCCCCGGGG**TACCCT**CCTTCCCGGGAGGCGCGCCTCCC**GAGGAGAA**CGGTACCC**ATG**..	VV1-2
	(SEQ ID NO: 5)
TTGACAAGGGAAAGCCGGGGTGC**TAACTT**AGGGATTGCGCTGCCCT...	VV57
....ATACGTAGCT**AGCCCCGG**CCGGCCTAGG**ATG**...	(SEQ ID NO: 6)
TTTATTCGCAAAGCCCCCCGGTGC**TATAAT**GGAAGACGGCGTCTAAACGCCTTCT**AGGAGCGC**T**ATG**...	VV34
	(SEQ ID NO: 7)
TTGACGCTCCCCCAAAAGCCCCCT**TATAAT**CGCTGTGGAATAGCTTCC**AAAGGAGG**TACGGT**ATG**...	VV40
	(SEQ ID NO: 8)
TTGTAGAGGCGGCGCTCCGCCTC**TATGGC**CACCCGGATCATTTACCCCCTCATCAAGGCCACC...	VV37
	(SEQ ID NO: 9)
TTGACAAAGGCCATGCCTCCTTGG**TATCTT**CCCTTTTGCGCTGCCCTGAGGGGG...	VV53
	(SEQ ID NO: 10)
TTGACAAGGTCTTCCGCCAGGC**CTCCAT**CCACCACGTCATCGTCCTGGAG...	VV18
	(SEQ ID NO: 11)
TTCGAATCCCTCCGGGCCCGCCATTGT**TATCTT**GGAAATGGGTAGCCTTT...	VV51
	(SEQ ID NO: 12)

ATG Start Codons shown in bold font are verified real start codons as identified by "CodonUse" which looks for codon usage patterns in open reading frames. "clone" denotes promoter clone name. Putative SD, translation start, –35 and –10 sites are shown in bold.

To determine the promoter activity in each of the clones, a Tbg assay was performed, as shown in **Table 4** below. As shown therein, expression from the promoters varies.

Table 4

Expression characteristics of the cloned promoter candidates

Promoter Clone	E. coli Average Units	Temp. Induction 42°C/30°C *	Homologies &Comments
VV1	186 ± 9	1.1	
VV2	36 ± 24	1.6	none
VV4	152 ± 23	1.4	*Thermus* NADH dh, DNA pol
VV7	120 ± 39	1.2	none-
VV12	132 ± 23	1.4	
VV13	350 ± 13	2.1	
VV15	150 ± 8	1.7	glutamate synt, *Thermus dh, def, fmt, proC*
VV18	97 ± 2	1.3	
VVHC1	287 ± 16	1.6	
VV31	5836 ± 511	1.6	
VV32	67 ± 15	1.4	*B. sp wap, licT*
VV33	87 ± 20	1.0	
VV34	66 ± 21	1.5	23S rRNA***
VV35	81 ± 16	1.3	fus???
VV36	40 ± 12	1.3	Ile-tRNA synthetase
VV37	62 ± 8	1.4	*Thermus* slp, nox, pol, Zea rbd inac
VV38	59 ± 9	1.6	*Thermus* lysyl tRNA synthetase
VV39	65 ± 6	1.4	ribosomal spacer
VV40	118 ± 10	1.2	*mus munculus* transcription factor
VV47	95 ± 24	1.1	
VV51	210 ± 4	1.4	
VV53	149 ± 33	2.0	
VV57	56 ± 27	2.1	T. thermophilus Chaperonin
VV70	134 ± 11	1.1	T. thermophilus ribonucleaseH
VV1.2	2000 ± 242	nd	Tbg with promoter

Assays were performed as described for ß-galactosidase in Miller (Miller, J. H. A short course in bacterial genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor. 1992) using o-nitrophenyl galactopyranoside (ONPG) as a substrate modified to be run at 65°C to assay for Tbg. *Ratio of expression from *E. coli* host grown at 42°C divided by *E. coli* host grown at 30°C.

The promoters were then assayed at higher temperatures in *E. coli*. While a temperature sensitive repressor cloned along with the promoter was unexpected, although possible, temperature dependence of the promoter could potentially be observed because DNA from *Thermus* is typically GC rich. In addition, it was also possible that the promoter would cross-react with *E. coli* regulatory elements. Three of the promoter clone candidates showed twice the level of expression at 42°C. Clone VV57 appears to have homology to a *Thermus* heat shock protein (actually a chaperonin) as is shown in **Fig. 3**.

Example 2

Integrative promoter-identification vector for Thermus

To evaluate strength of the identified core promoter regions in *Thermus*, a novel integrative promoter test vector was constructed for use in *T. flavus*. The vector was constructed to include a thermostable kanamycin resistance gene (kan^{tr2}), which had been previously demonstrated to function in *Thermus* . The vector integrates by a double-crossover event so the insertion is stable and permanent. The integrative vector $pTG100kan^{tr2}$ for use in *T. flavus* is a suicide vector having the Km^R gene as a selective marker, and a *leuB* as a region of homology where integration into the chromosome occurs (Weber, et al. (1995) A chromosome integration system for stable gene tranfer into *Thermus flavus*. *Bio/Technology*, Vol. 13(3): 271-275). Other such reporter genes and insertion sites could be used as well.

A promoter-test vector was redesigned from $pTG100kan^{tr2}$ (Weber, et al. (1995) A chomosome integration system for stable gene tranfer into *Thermus flavus*. *Bio/Technology*, Vol. 13(3): 271-275). In a novel vector, pTG200, the promoterless Km^R gene was utilized as a reporter gene (**Figure 3B**). In this vector, Km^R is oriented in the opposite direction to *leuB*. Therefore, upon integration of a fragment bearing a promoter in front of Km^R into the *T. flavus* chromosome, simultaneous transcription from the *leu* and Km^R promoters might cause occlusion transcription, which is a phenomenon observed when transcription through promoter inhibits promoter's function. To avoid such interference, the *Thermus* transcriptional terminator was inserted downstream of the Km^R gene. A consensus sequence of *Thermus* transcriptional terminator was derived by analysis of a number of *Thermus* terminators as shown in **Figure 3A**. In this example, the sequence below was used as a terminator (underlined sequences signify the regions of inverse homology):

TGCCACCCCATGCTGGCTTGCGCCAGCATGGGGGCCCCGGCAAAAGAATTC
(SEQ ID NO. 13)

Positioning the promoterless Km^R gene in the opposite direction to *leuB*, pTG200 did not confer Km^R to *T. flavus* cells and, therefore, could be used as a promoter probe vector.

The terminator sequence was derived by a comparison of terminators shown in Figure3A and using the *his* terminator as a model. A terminator sequence was obtained as a part of a larger fragment amplified by PCR using the following primers:

TR5KAN - Containing NcoI site, transcriptional terminator (underlined), EcoRI site, and 5' end sequence of the KmR gene:

5'-acacacacacaCCATGGcctaa TGCCA CCCCATGCTGGCTTGC
NcoI ----------terminator----------

GCCAGCATGGGGGCCCCGGCAAAA GAATTCaaagggaatgagaatagtgaatggacc-3'
----------terminator---------- EcoRI ---------5'end of KmR gene----
(SEQ ID NO: 14)

KAN3 - Containing PstI and HindIII sites and 3' portion of the Km gene

5'-gagcatggccCTGCAG AAGCTT caaaatggtatgcgttttgacacatcca-3' (SEQ ID NO: 15)
PstI HindIII ------3'end of KmR gene----

A DNA fragment obtained by PCR amplification of the KmR gene from pTG100 was digested with PstI and NcoI and subcloned into the pTG100 cleaved with NcoI and NsiI.

It turned out that the terminator diminished transcription but did not terminate it completely so that we could observe weak growth of *T. flavus* on Kmtransformed with the plasmid described above. To avoid this effect, we inverted the kanamycin gene in the construct. To invert the gene, we amplified it by PCR using primers 3KM-RI and 5KM-H3 to obtain a DNA fragment bearing promoterless KmR gene:

5'-acacacGAATTCcaaaatggtatgcgttttgacacatcc-3' (SEQ ID NO: 16)
EcoRI

5'-cacacacaAAGCTTtacgtatctagagggaatgagaatagtgaatggacc-3' (SEQ ID NO: 17)
HindIII

The fragment was cleaved with EcoRI and HindIII and subcloned into the described above plasmid cleaved by the same enzymes to give pTG200. The resulting plasmid has a promoterless KmR gene with a unique HindIII site upstream and a terminator downstream. We did not observe growth of *Thermus* cells transformed with pTG200 on Km plates. Hence, the plasmid can be used as a promoter probe vector in *Thermus*.

To check activity of the promoters identified in *E. coli*, the core regions attached to the kanamycin resistance gene were amplified by PCT using primer 3KM-RI and one of the following primers:

VV37KM
acacacAAGC Ttgtagaggc ggcgctccgc ctctatggcc acccggatca tttacccct catcaaggag gagaatagtg Aatggaccaa taatgac (SEQ ID NO: 18)

VV53KM
acacacacAA GCTTgacaaa ggccatgcct ccttggtatc ttccctttg cgctgccctg aggaggagaa tagtgaatgg accaataata atgact (SEQ ID NO: 19)

VV18KM
acacacacAA GCTTgacaag gtcttccgcc aggcctccat ccaccacgtc atcgtcctgg aggaggagaa tagtgaatgg accaataata atgact (SEQ ID NO: 20)

VV51KM
acacacAAGC Ttcgaatccc tccgggcccg ccattgttat cttggaaatg ggtagccttt aggaggagaa tagtgaatgg accaataata atgact (SEQ ID NO: 21)

VV57KM
acacacacAA GCTTgacaagg gaaagccggg gtgctaactt agggattgcg ctgccctcat acgtaggagg agaatagtga atggaccaat aataatgac (SEQ ID NO: 22)

VV12D2 PRIMER
acacacacAA GCTTgacatt ccccccgccc cgccgtaccct ccttcccggg aggaggagaa tagtgaatgg accaataata atgactag (SEQ ID NO: 23)

All PCR reactions were performed in a volume of 100 microliters. The reaction mixture contained 50 mM KCl, 10 mM TrisCl pH 8.3, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (A, C, G, T), 2 U Taq DNA polymerase (Perkin-Elmer), 40 pmole of each primer, 100 ng of template DNA. The thermalcycler repeated the following steps for 30 cycles: 1 minute at 94°C, 1 minute at 55°C, 1 minute at 72 °C. PCR fragments were cleaved by HindIII and EcoRI and subcloned into pTG200 cleaved by the same enzymes. DNA of the resulting plasmids was used to transform *T. flavus*. Transformed cells were plated on LB agar containing 20 µg/ml Km.

Promoter VV1-2 was also modified by removal of the larger or both inverted repeats because it appeared that this inverted repeat might effect ribosome binding. Putative core promoters were placed immediately upstream of the Km^R gene and integrated into the *T. flavus* chromosome. Promoters VV1-2/D2, VV40, VV53, and VV57 proved functional in *T. flavus*, conferring Km resistance to the cells upon transformation at 20µg/ml. It is possible that the other promoters may confer resistance below this level, however as the level of kanamycin drops to about 10 µg/ml, background growth of *Thermus* begins to occur. The unmodified VV1-2 *without* the inverted repeat removed *did not* give expression when tested, in contrast to VV1-2/D2 which gave the strongest expression.

VV1-2 **TTGACATTCCCCCCGCCCCGGGGTACCCTCCTTCCCGGGAGGCGCGCCTCCCGAGGAGAA (SEQ ID NO: 24)**

VV1-2 /D2 **TTGACATTCCCCCCGCCCCGGGGTACCCTCCTTCCCGGGAGGAGGAGAA (SEQ ID NO: 25)**

Figure 4. Removal of hairpin loop region in VV1-2 promoter. The –35, -10 and SD regions are shown in bold. The inverted repeat is underlined.

To confirm integration of the Km^R gene into the *T. flavus* chromosome had occurred in the strains from transformants were obtained, Southern hybridization and PCR analysis of the promoters was performed. The data indicates that integration of Km^r into *leuB* had occurred. To estimate activity of the promoters, clones were cultured on TT agar plates

containing various amounts of Km. The data indicated that the modified VV1-2 was the most active of the tested promoters, conferring to the cells resistance of up to 1000 μg/ml Km (**Table 5**). While high levels of kanamycin resistance had been reported on multicopy plasmids in other organisms, stable resistance to kanamycin at these levels had not been previously observed in *Thermus*.

Table 5

Ability of the tested promoters to confer kanamycin resistance on a promoterless kantr2 gene in Thermus

Plasmid-promoter	Initial Transformation at 20µg/ml Kanamycin	Kanamycin resistance at higher levels*						
		50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml	600 µg/ml	800 µg/ml	1000 µg/ml
pTG200-none	-							
pTG200-VV1-2	-							
pTG200-VV1-2/D2	+	+	+	+	+	+	+	+
pTG200-VV57	+	+	+	+	-	-	-	-
pTG200-VV34	-							
pTG200-VV40	+	+	+	+	+	+	+	-
pTG200-VV37	-							
pTG200-VV53	+	+	+	+	+	+	-	-
pTG200-VV18	-							
pTG200-VV51	-							

*The transformants were tested on TT-Agar plates with the corresponding amounts of kanamycin added.

Example 3

Integrative promoter-identification vector for Thermus

Promoters are selected by direct cloning and of libraries into a *Thermus* strain as well. This method avoids the initial characterization of promoter activity in *E. coli*. Construction of a *Thermus* promoter library for direct transformation in *Thermus* is carried out utilizing a screening or selection marker that functions in *Thermus*. This marker is incorporated into a promoter probe vector capable of either integrating into the *Thermus* chromosome or being maintained extrachromosomally on a suitable plasmid in *Thermus*. A vector such as the promoter-test vector pTG200 (described above, Example 2) is one such vector. In this case, the promoterless Km^R gene is utilized as a reporter gene (**Figure 3B**).

In order to use the promoter probe vector, *Thermus* chromosomal DNA is partially digested with a frequent-cutting endonuclease (such as *Sau*3AI) and a fraction of 1 kb fragments are purified by elution from an agarose gel. These fragments are then ligated with DNA from the promoter-test pTG200 vector which has been digested with an appropriate enzyme (*BclI*) for cloning. The ligated DNA is then used to transform *Thermus*. The plating media contains a suitable amount of the selection (or screening) agent such as kanamycin in this example, which is determined by testing a series of concentrations and choosing one just strong enough to prohibit growth of untransformed strains. This allows selection or screening for promoter sequences which activate the reporter gene.

To confirm integration of the Km^R gene into the *T. flavus* chromosome has occurred in the strains from which the transformants are obtained, Southern hybridization and PCR analysis of the promoters is performed. The nucleotide sequences of the putative promoter sequences is then determined by sequencing the *Thermus* genomic fragment. Computer analysis of these sequences using BLASTN search algorithm is then utilized to reveal putative core promoter regions. The promoter strength of the discovered promoters is then analyzed using the methods described in Example 2 using either the same or a different reporter gene.

Example 4

Putative promoters from T. flavus

To date, approximately 300 kb comprising about 20% of *T. flavus* genome has been sequenced by the applicants. A search for putative promoters within these sequences has been accomplished using Neural Network for Promoter Prediction software, NNPP. NNPP is a time-delay neural network consisting mostly of two feature layers, one for recognizing TATA-boxes and one for recognizing so called "initiators", which are regions spanning the transcription start site. Both feature layers are combined into one output unit.

Nineteen putative promoters were identified in the 25 kb contig by NNPP (**Table 6**). Though a basis for a search by NNPP is -10 and +1 regions, -35 boxes which were found for

most of identified promoters match consensus sequence TTGNCN derived from published data and from data obtained in the experiments described herein.

Table 6

Putative Promoter Sequences Predicted for T. flavus contig TF4-6-10.1

-35 -10

ATGGCA**TTGTCT**TTCCGCTATTGAATGAC**TATCAT**TCAAGTATGGAAAGA (SEQ ID NO: 26)

GAGG**TTGCTT**GGTTCCGGTGCACGAGTTC**TATTCT**GCCCAGGCCGTAGCG (SEQ ID NO: 27)

GAGTACG**TTGACC**AGCGCTCCCCGAAAGG**TATAAG**CGGGCACGTAAAGCC (SEQ ID NO: 28)

ACC**TTGTCG**TGCCTCGCCTTGAGGTAGAG**GAACAT**GGCGTAGGGCTCCTG (SEQ ID NO: 29)

CTAATC**TGGAAG**TAGGCCGGGTTCTTGGC**GATGAT**CTCCCACACCAGCAC (SEQ ID NO: 30)

GAC**TTGCAG**AAACTTTTGGTAACCTGCCA**TAGCTT**CTACCCTCCTCGTTC (SEQ ID NO: 31)

TTTGTTAAAGGAAGCGAGCTTTCCTCGCA**CATAAT**TCACCAGATTCAAAT (SEQ ID NO: 32)

GCTCGCTTCCTTTAACAAAGGTGATCCGG**TACTAA**AAAATCTGCAAGAGG (SEQ ID NO: 33)

AACACGCATCTGA**TTGGCA**GACCTTTTTCCAGAA**TATTGT**TGAAGACCGT (SEQ ID NO: 34)

TATGACCGTGGATGAAGTCAGTACCTGGCCGCGG**TCTTAT**GGGCACCTGG (SEQ ID NO: 35)

GCAATCAGA**ATGTCA**AGCAAAAATTGGAGTCGC**TCAAAA**TCCCCGACTCC (SEQ ID NO: 36)

CAGGTCTAGT**TTGGCG**ACGCGAGGCTCAAGGGAA**TACCGT**CCCGGACCGC (SEQ ID NO: 37)

TTGG**TTGGTG**TCTTCGGCCAGAAAAGG**GAAATA**ATCCCAGGTCATGCGCC (SEQ ID NO: 38)

AACTGGT**TTGAGG**CGGCGCTTCATCTCGTC**AAAGTC**CACCAATCCCGGCT (SEQ ID NO: 39)

GAAGTT**TTGTAG**CGAGACCCAAGAGAAAT**CATGAT**ATGAGTGTGGTACTT (SEQ ID NO: 40)

GGGAGGCCATC**TTGTCT**GGATTGTAGCACTTCCC**TATCCT**TAGCCCAAGG (SEQ ID NO: 41)

GTGCGCCTATT**TTGAGT**TCTGCTTCGTGGAGGAG**GAAGAT**GGCTAAGCCG (SEQ ID NO: 42)

ACCCCGGGGGG**TTGACG**CACACCCCCCGATCTGC**TAACTT**GGCCTTAAGT (SEQ ID NO: 43)

GACCAACAGCCA**TTGGCG**CAAAGTACCACACTCA**TATCAT**GATTTCTCTT (SEQ ID NO: 44)

The putative promoter sequences of SEQ ID NO: 26-44 are amplified by PCR using primers containing restriction sites compatible with the restriction sites available for cloning 3' of the kanamycin sequence of the promoter-test vector pTG200. These fragments are then ligated with DNA from the promoter-test pTG200 vector which has been digested with an appropriate enzyme for cloning. The ligated DNA is then used to transform *Thermus*. The plating media contains a suitable amount of the selection (or screening) agent such as kanamycin in this example, which is determined by testing a series of concentrations and choosing one just strong enough to prohibit growth of untransformed strains. This allows selection or screening for promoter sequences which activate the reporter gene. To confirm integration of the Km^R gene into the *T. flavus* chromosome has occurred in the strains from which the transformants are obtained, Southern hybridization and PCR analysis of the promoters may be performed. Computer analysis of these sequences using BLASTN search algorithm may then be utilized to reveal putative core promoter regions. The promoter strength of the discovered promoters may then be analyzed using the methods described in Example 2 using either the same or a different reporter gene.

Example 5

Promoter Optimization for Gene Expression in a Thermophile

To optimize the promoters found to be useful in driving gene expression in a thermophilic organism, promoter deletions constructs are generated.

A. Promoter Optimization by PCR

One method to generate sequential deletions is by PCR amplification. A 1 kb thermophile genomic fragment that is observed to drive gene expression in a thermophilic organism is modified to generate subfragments at 100 nucleotide intervals by PCR. PCR primers are designed that correspond to regions of the 1 kb fragment as follows: bp 1-900, bp 1-800, bp 1-700, bp 1-600, bp 1-500, bp 1-400, bp 1-300, bp 1-200, and bp 1-100. The primers also incorporate restriction enzyme sites suitable for insertion into pTG200 reporter vector.

B. Promoter Optimization using an Exonuclease

Exonucleases such as Exo III or Bal31 may also be used to generate sequential deletions of the promoter regions. These enzymes are employed using standard molecular biology techniques or as described by supplier of these enzymes (New England Biolabs) to generate a series of random deletions by reacting with a linearized plasmid or fragment of DNA. The exonucleases generate a time-dependant set of deletions from one or both ends of the linear DNA. In the case of Bal31, deletions at both ends of the linear fragment that has been treated are obtained and the deleted promoter sequence subcloned into an appropriate test plasmid such as pTG200. In order to do this, the deletion ends are repaired with Mung Bean Nuclease so that they are suitable for subcloning, digested with an appropriate restriction endonuclease which can be used to isolate the remaining promoter fragment, and religating into the test plasmid. In the case of exo III, it is possible to obtain deletions at only one end (for example the end of the linearized plasmid containing the promoter sequence) if approprate set of restriction endonucleases are used. Several kits are available to do this (such as the Exo-Size™ kit from New England Biolabs). Exo III prefers digestion of DNA which contains a blunt or 3' recessed ends, so if two appropriate endonucleases can be used to linearize the plasmid, then after repairing the deleted ends to make them suitable for cloning with Mung Bean Nuclease and optionally adding a linker DNA, the DNA can be directly religated and used for testing without the need for subcloning. The DNA from either method can be transformed into *E. coli* and a series of plasmids containing a series of deletion constructs from each end of the putative promoter can be identified.

C. Testing the Constructs

Following promoter modification as described above, constructs containing one or more of the various subfragments are made using standard molecular biology techniques such

that each subfragment has the potential to drive expression of the kanamycin resistance gene following transformation of the thermophile with the ligated reporter vector. The thermophilic organism is then assayed for kanamycin resistance by plating on kanamycin-containing plates. Growth in the presence of increasing amounts of kanamycin indicates that the subfragment is active in the thermophilic organism.

Following identification of those subfragments having activity in the thermophile, the fragments may be combined by ligation or PCR amplification and co-inserted into pTG200 or another reporter vector. The ability of these combined subfragments to drive gene expression in the thermophile is then determined by the presence or absence of growth in the presence of kanamycin. In this manner, it is possible to identify those fragments that function additively or synergistically to drive high expression in the thermophile.

CLAIMS

We claim:

1. An isolated, recombinant DNA molecule for identification of a regulatory region of a thermophile genome comprising:

a) a reporter sequence;

b) a putative thermophile promoter operably linked to said reporter sequence to form a promoter / reporter cassette;

c) a drug resistance marker; and,

d) a 3' and a 5' DNA targeting sequence that are together capable of causing integration of at least a portion of said DNA molecule into the genome of a thermophile;

wherein said promote/reporter cassette is flanked by said 3' and said 5' DNA targeting sequences; and, said promoter/reporter cassette is positioned in the opposite orientation of the DNA targeting sequences.

2. A recombinant DNA of claim 1 wherein said reporter sequence is *tbg*.

3. A recombinant DNA of claim 1 wherein said reporter sequence is *lacZ*.

4. A recombinant DNA of claim 1 wherein said drug resistantce marker confers upon a thermophile resistance to kanamycin.

5. A recombinant DNA of claim 1 wherein said putative promoter sequence is a fragment of the genome of a thermophile.

6. A recombinant DNA of claim 5 wherein said fragment was isolated following limited digestion of the genome with a restriction enzyme.

7. A recombinant DNA of claim 1 wherein said thermophile putative promoter sequence was isolated from a bacteria of the genus *Thermus*.

8. A recombinant DNA of claim 1 wherein said thermophile putative promoter sequence was isolated from the thermophile *Thermus flavus*.

9. A recombinant DNA of claim 1 wherein said thermophile putative promoter sequence was isolated from the thermophile *Thermus thermophilus*.

10. A method of identifying a thermophile promoter comprising transforming a thermophile with a recombinant DNA molecule of claim 1 and detecting expression of the reporter sequence.

11. A method of claim 10 wherein said thermophile is from the genus *Thermus*.

12. A method of claim 10 wherein said thermophile is *Thermus flavus*.

13. A method of claim 10 wherein said thermophile is *Thermus thermophilus*.

14. An isolated DNA molecule comprising a promoter sequence identified by the method of claim 10.

15. An isolated DNA molecule comprising a thermophile promoter sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID

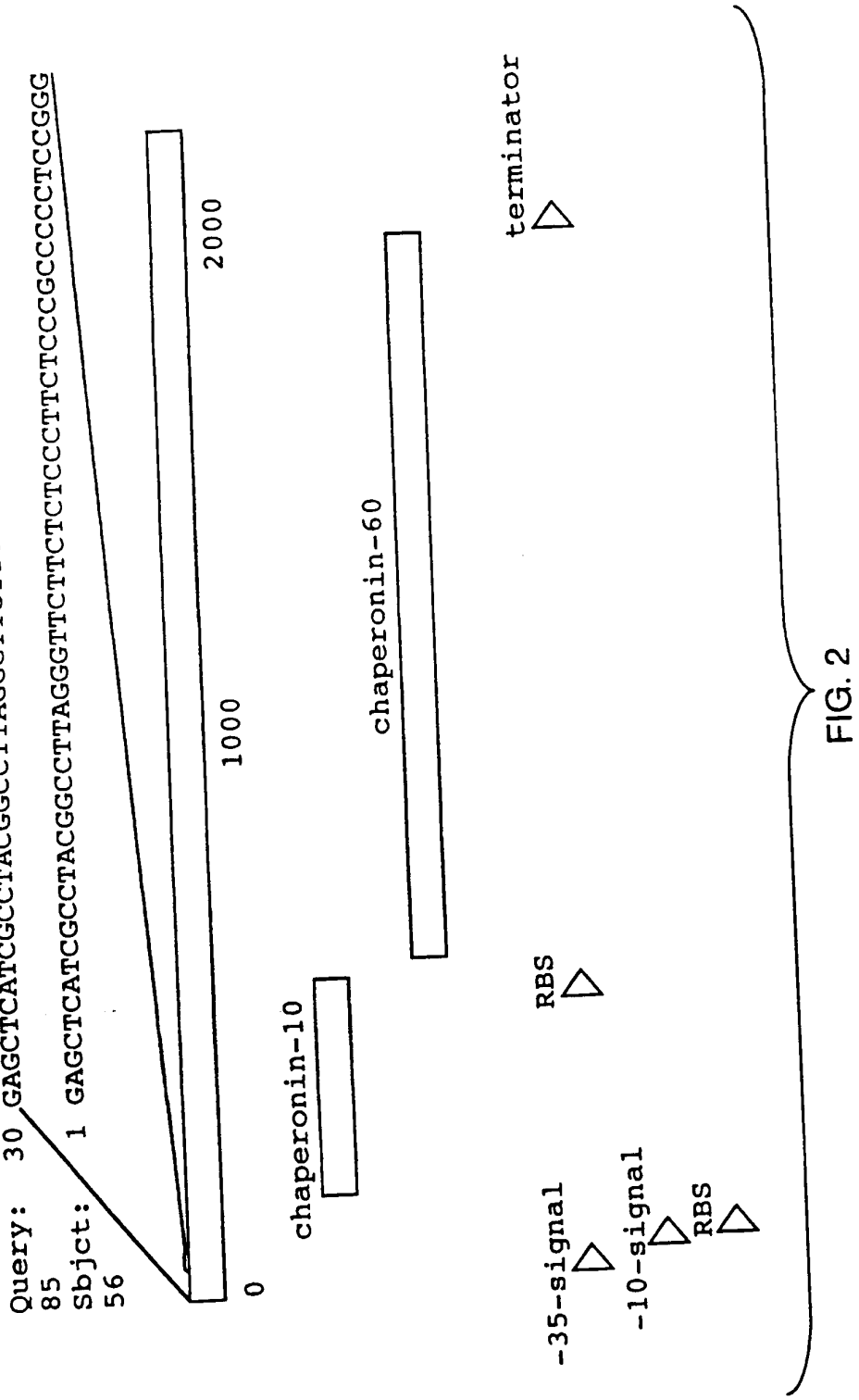
Query: 30 GAGCTCATCGCCTACGGCCTTAGGGTTCTTCTCTCCCTTCTCCCGCCCCCTCCGGG
85
Sbjct: 1 GAGCTCATCGCCTACGGCCTTAGGGTTCTTCTCTCCCTTCTCCCGCCCCCTCCGGG
56
0
1000
2000
chaperonin-10
chaperonin-60
terminator
RBS
-35-signal
-10-signal
RBS

FIG. 2

Comparison of Terminators from *Thermus*.

```
<leu Termin.    TCCCCAGGA ACCTT:TTGC CGGGGCCCCC ATGCTGGCGC AAGCCAGCAT GGGGTGGTGT TACAGGTGCC GCAGA
<his terminator     CAGGG ACCTT:TTGC CGGGGCCCCC ATGCTGGCGC AAGCCAGCAT GGGGTGGCAT CAAAGCACCC C
<icd terminator               CCATGC C:GGG:CCCC ATGCCGGCCC AAGCCGGCAT GGGGTGGCCT TA
<proC Terminator            CCTTC:TGC CGGGGCCCCC ATGCGGGCGC AAGCC:G:AT GGGGTGGCCT CA
<phe S/T Terminator    GG GCCTT:TTGC CGGGGCCCCC ATGCTGGCGC AAGCCAGCAT GGGGTGGCAT TA
<pol Termin.    TTCCCCAGAA ACCTT:TTGC CGGGGCCCCC ATGCTGGCTT GGGCCAGCAT GGGGTGGTAT CA
```

FIG. 3A

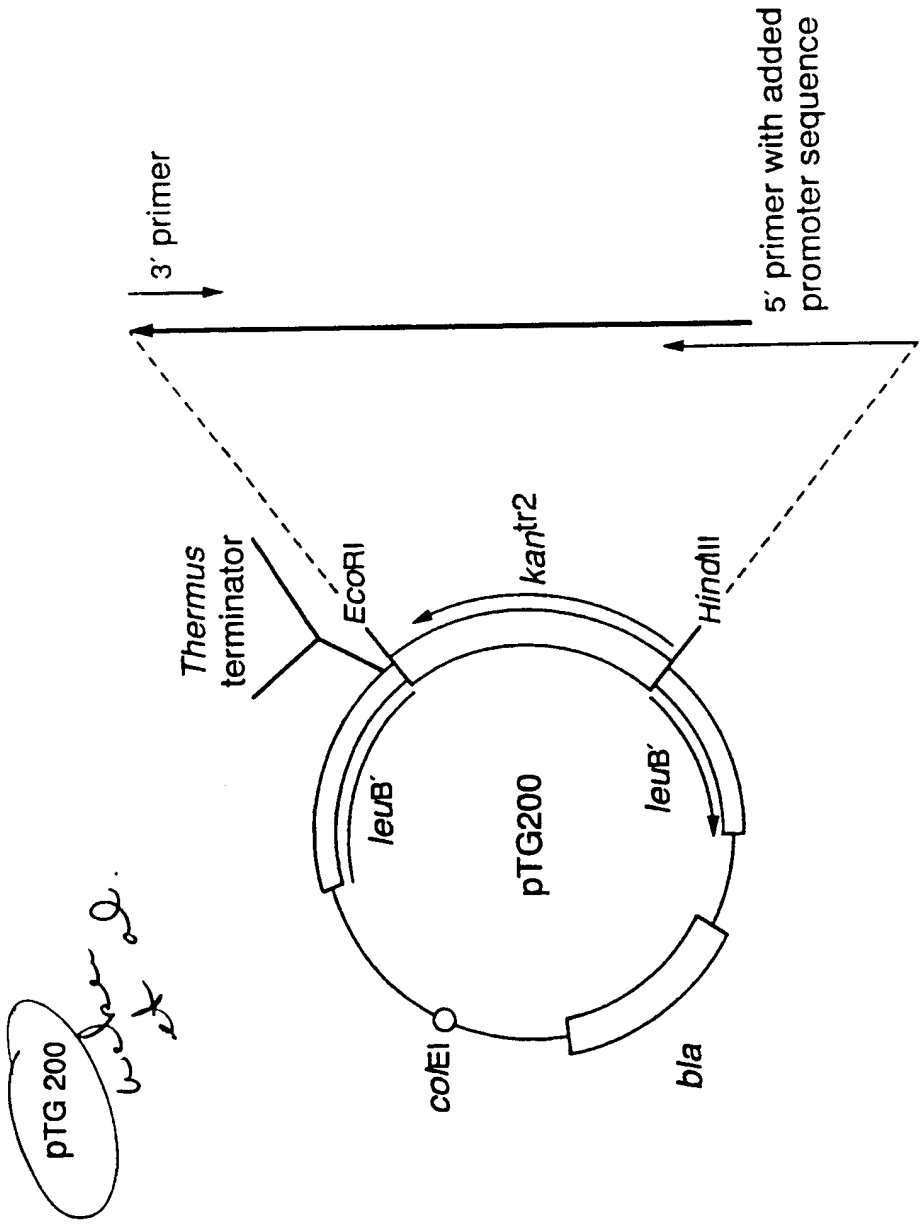
3' primer
5' primer with added promoter sequence
Thermus terminator
EcoRI
kantr2
HindIII
leuB'
pTG200
leuB'
colEI
bla
pTG 200

FIG. 3B

SEQUENCE LISTING

<110> Peredeltchouk, Mikhail
Vonstein, Veronika
Demirjian, David

<120> Thermus Promoters for Gene Expression

<130> 99-559

<140> 09/390,867
<141> 1999-09-07

<160> 44

<170> PatentIn Ver. 2.0

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ggcctaggat ggcaattatt caatttc 87

<210> 2
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
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<210> 3
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<220>
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<210> 4
<211> 88
<212> DNA
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<220>
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ttctggctca aaatggtatg gttttgac 88

<210> 5
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<212> DNA
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<220>
<223> Description of Artificial Sequence: VV1-2 promoter sequence

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cggtacccat g 71

<210> 6
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<400> 6
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cggccggcct aggatg 76

<210> 7
<211> 67
<212> DNA
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<220>
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tttattcgca aagccccccg gtgctataat ggaagacggc gtctaaacgc cttctaggag 60
cgctatg 67

<210> 8
<211> 65
<212> DNA
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<220>
<223> Description of Artificial Sequence: VV40 promoter sequence

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gtatg 65

<210> 9
<211> 63
<212> DNA
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<220>
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TR5KAN

<400> 14
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<210> 15
<211> 50
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<220>
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KAN3

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<210> 16
<211> 39
<212> DNA
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<220>
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3KM-RI

<400> 16
acacacgaat tccaaaatgg tatgcgtttt gacacatcc 39

<210> 17
<211> 50
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<220>
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5KM-H3

<400> 17
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<211> 97
<212> DNA
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7KM

<400> 18
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<210> 19
<211> 96

<212> DNA
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VV53 KM

<400> 19
acacacacaa gcttgacaaa ggccatgcct ccttggtatc ttccctttg cgctgccctg 60
aggaggagaa tagtgaatgg accaataata atgact 96

<210> 20
<211> 96
<212> DNA
<213> Artificial Sequence

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VV18 KM

<400> 20
acacacacaa gcttgacaag gtcttccgcc aggcctccat ccaccacgtc atcgtcctgg 60
aggaggagaa tagtgaatgg accaataata atgact 96

<210> 21
<211> 96
<212> DNA
<213> Artificial Sequence

<220>
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VV51 KM

<400> 21
acacacaagc ttcgaatccc tccgggcccg ccattgttat cttggaaatg ggtagccttt 60
aggaggagaa tagtgaatgg accaataata atgact 96

<210> 22
<211> 100
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tacgtaggag gagaatagtg aatggaccaa taataatgac 100

<210> 23
<211> 89
<212> DNA
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<400> 23

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atagtgaatg gaccaataat aatgactag 89

<210> 24
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: VV1-2 promoter sequence

<400> 24
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<210> 25
<211> 49
<212> DNA
<213> Artificial Sequence

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<400> 25
ttgacattcc ccccgccccg gggtaccctc cttcccggga ggaggagaa 49

<210> 26
<211> 50
<212> DNA
<213> Artificial Sequence

<220>
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<400> 26
atggcattgt ctttccgcta ttgaatgact atcattcaag tatggaaaga 50

<210> 27
<211> 50
<212> DNA
<213> Artificial Sequence

<220>
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gaggttgctt ggttccggtg cacgagttct attctgccca ggccgtagcg 50

<210> 28
<211> 50
<212> DNA
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<400> 28
gagtacgttg accagcgctc cccgaaaggt ataagcgggc acgtaaagcc 50

<210> 29
<211> 50
<212> DNA
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<400> 29
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<210> 30
<211> 50
<212> DNA
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ctaatctgga agtaggccgg gttcttggcg atgatctccc acaccagcac 50

<210> 31
<211> 50
<212> DNA
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gacttgcaga aacttttggt aacctgccat agcttctacc ctcctcgttc 50

<210> 32
<211> 50
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<223> Description of Artificial Sequence: Putative promoter sequence

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tttgttaaag gaagcgagct ttcctcgcac ataattcacc agattcaaat 50

<210> 33
<211> 50
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Putative promoter sequence

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<210> 34
<211> 50
<212> DNA
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<211> 50
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<210> 38
<211> 50
<212> DNA
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<212> DNA
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<211> 50
<212> DNA
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<210> 43
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<212> DNA
<213> Artificial Sequence

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<211> 50
<212> DNA
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